PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, A01H 4/00 **A2**	**(11) International Publication Number:** WO 94/02620
	(43) International Publication Date: 3 February 1994 (03.02.94)

(21) International Application Number: PCT/US93/07009

(22) International Filing Date: 26 July 1993 (26.07.93)

(30) Priority data:
07/920,409   27 July 1992 (27.07.92)   US

(71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(72) Inventors: TOWNSEND, Jeffrey, A. ; 2988 N.W. 132nd Court, West Des Moines, IA 50325 (US). THOMAS, Laurie, A. ; 655 38th Street, Des Moines, IA 50312 (US).

(74) Agents: PEARLMUTTER, Nina, L. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

---

(54) Title: AN IMPROVED METHOD OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CULTURED SOYBEAN CELLS

(57) Abstract

A method for producing transgenic soybean plants is disclosed. The method employs conditions necessary for genotype-independent, *Agrobacterium*-mediated transformation of soyben explants and utilization of specialized medium to cause root induction.

AN IMPROVED METHOD OF AGROBACTERIUM-MEDIATED
TRANSFORMATION OF CULTURED SOYBEAN CELLS

5

10   Field of the Invention

The present invention relates to the production of
transgenic soybean plants through Agrobacterium-mediated
transformation.

15

                        Background of the Invention

Soybean (Glycine max) is one of the world's most important
crop plants. It is an annual seed legume grown on more than 50
20 million hectares for the production of oil and protein. The
value of the crop is estimated to be over 20 billion dollars.
Each year more than 100 million metric tons of soybean are
produced. The development of gene transfer techniques for
leguminous plants is of commercial interest because it
25 facilitates the development of new cultivars with improved
disease resistance, tolerance to specific herbicides and
increased nutritional value. However, molecular approaches for
the improvement of soybean are limited by the technology
available to produce transgenic soybean plants.
30   Many plants, including several important crop plants, have
been genetically modified using Agrobacterium-mediated gene
transfer. McCormick, et al. (1986) Plant Cell Rep. 5, 81-84;
Radke, et al. (1988) Theor. Appl. Genet. 75, 685-694; Umbeck,
et al. (1987) Bio/Technology 5, 263-266; Botteman and Leemans
35 (1988) Trends in Genetics 4, 219-222. Unfortunately, even
though some genotypes of these dicotyledonous species are
susceptible to Agrobacterium infections (Facciotti et al.,
(1985) Biotechnology 3, 241-246; Owens and Cress (1985) Plant
Physiol. 77, 87-94; Byrne et al. (1987) Plant Cell Tissue and
40 Organ Culture 8, 3-15), the use of Agrobacterium for
transformation is limited due to the lack of available and

efficient transformation and regeneration procedures.

Until recently soybean was considered to be outside the host range of Agrobacterium. DeCleene and DeLey (1976) Bacterial Rev. 42, 389-466. Later reports suggested that
5 soybean has a limited susceptibility to Agrobacterium which is soybean genotype and Agrobacterium strain dependent. Byrne, et al. (1987) Plant Cell Tissue Organ Cult. 8, 3-15. Only one variety of soybean (Peking) has been successfully transformed, and further, this variety is of no commercial value. Few
10 transgenic soybean plants of this transformable variety have been produced by cocultivation of regenerable explants with Agrobacterium. Hinchee, et al. (1988) Bio/Technology 6, 915-922. For transformation of soybeans via Agrobacterium-mediated methods to become commercially viable, the method must
15 permit direct transformation of elite commercial cultivars.

In addition to these problems, soybean is known to be the most difficult Glycine species to regenerate. Explants do not readily produce roots, making further cultivations of transgenic material almost impossible. Thus, the methods used
20 for transformation and regeneration remained unreliable and ineffective until now.

Therefore, it is an object of this invention to provide an improved method for Agrobacterium species to infect plant cells and to transfer T-DNA to the plant cells for expression
25 therein.

It is another object of this invention to provide a novel induction medium for Agrobacterium species for enhancing the capacity of said species to infect and transform soybean plant cells.
30 Still another object of this invention is to provide novel growth media and methods for the regeneration of soybean explants into whole plants.

The process of this invention represents a great improvement in the techniques of Agrobacterium-mediated
35 transformation in soybean tissue and its regeneration into transgenic soybean plants.

Summary of the Invention

In accordance with this invention, there is provided a a simple, rapid, and reliable process for the production of transgenic soybean plants. The method is effective with all
5 soybean varieties including elite commercial cultivars, and shows a substantial improvement over previously-used systems because it provides necessary and hereto-missing factors for transformation and regeneration of soybeans, and optimizes these and other factors for the successful production of
10 healthy, fertile transgenic plants.

This invention comprises a genotype-independent method for producing a transgenic soybean plant comprising:

(a) cocultivating an explant derived from hypocotyl or cotyledonary nodes of a germinated soybean seed with
15 Agrobacterium species containing a chimeric gene in the presence of a signal compound selected from the group consisting of acetosyringone, α-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid, and sinapinic acid and mixtures thereof;
20   (b) inducing virulence of the Agrobacterium by coculture at a temperature below 28° C; and

(c) inducing virulence of the Agrobacterium by decreasing the pH of the plant culture media below pH 6.0.

This process involves the coculture of soybean hypocotyl
25 or cotyledonary node explants with an Agrobacterium species carrying a plasmid into which is inserted the gene or genes of interest. A soybean explant is a piece of soybean tissue that is taken from a donor plant and is capable of producing callus in culture. Soybean hypocotyl tissue is that portion of the
30 stem of a soybean plant embryo or seedling below the cotyledons and above the root. A cotyledon is an embryonic leaf, and a cotyledonary node is that part of the seedling between the embryonic axis and the cotyledons which botanically defines the division of the hypocotyl and the epicotyl (the embryonic
35 shoot).

Several factors which significantly impact the transformation of cultured soybean cells have been identified in arriving at this invention. The most important of these

appears to be the induction of the virulence (vir) genes in Agrobacterium by proper use of signal molecules during cocultivation. Cultured soybean cells lack or have a limiting amount of the necessary signal molecules to initiate the
5 transformation process. These results are in general agreement with other studies which have recognized the importance of vir gene induction for soybean transformation but failed to solve the problem. Delzer, et al. (1990) Crop Sci. 30, 320-322; Owens and Smigocki (1988) Plant Physiol. 88, 570-573. This invention
10 uses acetosyringone, a phenolic compound produced by wounded plant cells, to induce the vir genes. Other phenolic compounds, α-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid, and sinapinic acid, also induce the vir genes and their use individually or in combination with
15 acetosyringone can result in improved soybean transformation efficiencies. Use of adequate amounts of signal molecules in the cocultivation process has in every instance resulted in enhanced transformation frequency.

The temperature for cocultivation was discovered to be
20 another important factor. The usual temperatures (26-28°C) for the culture of soybean cells are inappropriate for efficient transformation. A lower temperature resulted in highly effective transformation. The inhibition observed at 28° C. did not seem to result from overgrowth of bacteria and
25 consequent reduction in plant cell viability at higher temperatures, because explants cocultured at both temperatures produced voluminous callus when kanamycin sulfate was excluded from the media. Media pH during coculture also affects transformation rate. Measures such as buffering at low pH (pH
30 5.5) ensure high efficiency. While not intending to be limited by theory, it is believed these improved efficiencies are related to signal molecule response operation. It is known that vir gene induction is temperature and pH dependent with an optimum at approximately 20°C and pH 5.5. Alt-Moerle, et al.
35 (1988) Mol. Gen. Genet. 213, 1-8.

The successful transformation of soybean cells was also dependent upon the concentration of bacteria in the inoculum. In general, higher numbers of bacteria resulted in more

transformation events. Although a saturating concentration of bacteria may exist, above which no additional transformation events are produced, for soybean cells, this number appears to be very high. Our experiments suggest that the number may be approached by a single 30 minute inoculation with bacteria at or exceeding 3×10^8 viable cells/ml, and that conventional concentrations of 3×10^7 cells/ml (Deblaere, et al. (1987) Meth. Enzymology 153, 277-293.) are far too low.

However, in our work, a truly saturating condition for inoculation could not be identified. The sequential inoculations demonstrated that transformable soybean cells were not being targeted even when inoculations were conducted with very high concentrations of bacteria. One problem encountered when attempting to inoculate with high concentrations of bacteria was the tendency for aggregation of the bacterial cells. Aggregation resulted in a reduction in the number of bacteria available for attachment to plant cells. Aggregation began immediately upon resuspension of sedimented bacteria, and the rate of aggregation was concentration dependent. It was also dependent on the degree of mechanical mixing of the media. The composition of the cocultivation media (in which the bacteria are resuspended) can also influence aggregation. Therefore, only complete plant cell media appropriate for the induction of rapid division of plant cells and high level induction of the Agrobacterium vir genes were used in this process.

Substantial increases in transformation frequency were achieved using inoculation conditions which limited the aggregation phenomenon and accounted for its effects. First, inoculations were conducted with high initial bacteria concentrations at room temperature with a minimum of mixing. Second, they were conducted in batches such that each explant was inoculated with a newly resuspended pellet of Agrobacterium in log growth phase. The wounding of the explant was done in the inoculum, in the presence of the highest possible concentration of available bacteria (i.e. before aggregation significantly reduced the number of independent cells).

Third, the period of inoculation was no less than thirty minutes, beginning when the last explant in a given batch had been wounded. Longer time periods did not result in enhanced transformation frequency. Sequential inoculation resulted in a modest increase in frequency. The increase was probably due to an increase in the number of bacteria available to transform the plant cells. From these experiments, it is clear that soybean cells can tolerate long periods of exposure to high concentrations of Agrobacterium.

By using conditions which assure vir gene induction and providing the necessary high concentration of Agrobacterium, we have for the first time been able to achieve consistently high frequencies of soybean transformation. The method of this invention produces these frequencies with several different strains of Agrobacterium and is only marginally affected by the original susceptibilities of soybean varieties to Agrobacterium infection.

After transformation, explants are cultivated in liquid counterselection medium, then transferred to solidified selection medium, and the process is repeated as described in the art for identification by transgenic markers such as gus. McCabe, et al. (1988) Bio/Technology 6, 922-926. Shoots are induced in transgenic explants by known methods. Wright, et al. (1986) Plant Cell Reports 5, 150-154; Barwale, et al. (1986) Planta 167, 473-481. The shoots are excised and, by the addition of pyroglutamic acid to a hormone-enriched growth medium, roots are readily induced. This is contrary to reports that roots are rarely induced from soybean tissue and then only in hormone-free medium. Whole mature, reproductive plants are produced after transfer to greenhouse culture in soil.

Detailed Description of the Invention

We have expressed the β-glucuronidase (gus) gene from Escherichia coli in soybean hypocotyl cells after cocultivation with Agrobacterium tumefaciens carrying the gus gene. Disarmed strains of Agrobacterium harboring binary plasmids with chimeric gus and neomycin phosphotransferase II (npt II) genes

were vectors for DNA transfer. After cocultivation, soybean hypocotyl explants from the commercial cultivars listed in Table 1 were cultured on callus induction medium (Hinchee, et al. (1988) Bio/Technology 6, 915-922) containing 100 μg/ml

5 kanamycin sulfate. Transformation events were scored three weeks after cocultivation when hypocotyl explants were assayed histochemically for gus activity. Transformation events were visualized with the aid of a dissecting microscope (10X magnification). They appeared as sectors of stained plant

10 cells. Stable integration of the gus gene into the soybean chromosome was suggested by the retention of gene activity in the sibling cell population within the stained sectors. Multiple, independent events could be detected on some explants. These were bounded on all sides by cells not

15 expressing the gus gene. Sets of explants were inoculated, cocultured, selected and histochemically assayed together. Each set was an independent experimental unit, the numbers of explants being sufficient to compensate for variation due to genetic difference (within a seed lot), explant orientation

20 (within a seedling) and explant size. Treatments were scored by recording the total number of events detected on the explants in them. Using this method only transformed cells capable of sustained division were scored. The measurements were clearly distinguished over background gus activity and any

25 residual bacterial contamination.

Signal molecules. Table 1 shows the results of an experiment in which hypocotyl explants of eight different soybean varieties were inoculated (30 minutes) and cocultured (3 days) in the presence and in the absence of acetosyringone (100 μM).

30 Inoculum was prepared by resuspending log phase Agrobacterium in liquid plant cell media buffered with 10 mM MES to pH 5.5 with or without acetosyringone at a final concentration of 3×10^8 viable cells/ml. Explants were prepared in the inoculum, held there for 30 minutes and then transferred to agar

35 solidified media for the 3 day cocultivation. Cocultivation was conducted at 22°C. After cocultivation, the explants were washed and transferred to solid media containing antibiotics (kanamycin) for selection of transformed plant cells and

counterselection of <u>Agrobacteria</u>.   Subsequent   culture was at 28°C.   Transformed   sectors   were   produced   only   when acetosyringone was added to the inoculation and cocultivation media.   Transformed sectors were produced on all of the

5 varieties. The average number of transformed sectors produced on each variety (48 explants) was 107.   The number of transformed sectors ranged from a low of 44 (variety Cartter) to a high of 153 (variety 9391). In subsequent experiments, a few transformed sectors were produced on explants which did not

10 see acetosyringone but the frequency of such events was too low to be accurately measured.

Inclusion of acetosyringone in the overnight bacterial culture and in a bacterial preculture period with plant media resulted in no   enhancement   of   transformation   frequency.

15 Similar results were obtained when the monosaccharide glucose was incorporated in the inoculation and cocultivation media (data not shown).   All subsequent experiments were conducted with   acetosyringone   included   in   the   inoculation   and cocultivation media at 100 μM.

20
Table 1.   Number of transformed sectors produced by
cocultivation of soybean hypocotyl explants with
and without acetosyringone.

	Acetosyringone(μM)	
Soybean genotype	0	100
Peking	0	147
Williams 82	0	115
Cartter	0	44
9273	0	79
9302	0	117
9341	0	115
9391	0	153
9582	0	87
Mean	–	107

<u>Temperature</u>. Table 2 shows the results of an experiment in which hypocotyl explants which had been inoculated in a common petri dish were divided randomly into two sets. The

45 sets (24 explants each) were cocultivated on solid media at

- 9 -

different temperatures, 22°C and 28°C for three days. After cocultivation all explants were cultured at 28°C. Twelve separate inoculations were conducted using variety 9341 with Agrobacterium at 3×10^8 viable cells/ml for thirty minutes. The
5 mean number of transformed sectors produced on 24 explants cocultured at 22°C was 109. The mean number of 28°C transformed sectors was 1.4. In this experiment there is a roughly 80 fold increase in transformation frequency resulting from cocultivations at reduced temperature. All subsequent
10 cocultivations were conducted at 22°C.

Table 2. Number of transformed sectors produced by
cocultivation of soybean hypocotyl explants at 22 and 28
degrees Celsius.
15

	Temperature(°C)	
Inoculation	22	28
1	101	1
2	109	2
3	134	0
4	97	2
5	127	1
6	102	1
7	100	1
8	84	1
9	112	4
10	118	3
11	100	0
12	124	1
Mean	109	1.4

35

pH. Table 3 gives the results of an experiment in which explants were inoculated and cocultured at different pH levels; 5.5, 5.75 and 6.0. Fourteen separate inoculations were made at each pH. Media was buffered with 10 μm MES to insure pH
40 stability. Coculture then proceeded on similarly buffered, solidified media for three days. The mean number of transformed sectors produced on 24 explants at pH 6.0 was significantly lower (203) than that produced at either pH 5.5 (286), or pH 5.75 (288). This is contrary to published results showing no
45 demonstrated effect of pH on Glycine max when using

- 10 -

acetosyringone. Godwin, et al. (1991) <u>Plant Cell Rep</u>. 9, 671-
675. All subsequent inoculations and cocultivations were
conducted with media adjusted to pH 5.5 and buffered with 10 μM
MES.

5

Table 3. Number of transformed sectors produced by cocultivation
of soybean hypocotyl explants at pH 5.50, 5.75 and 6.00.

	pH		
Inoculation	5.50	5.75	6.00
1	186	324	210
2	278	307	192
3	301	263	162
4	473	334	312
5	192	232	92
6	245	256	129
7	264	288	186
8	206	312	105
9	253	313	245
10	259	249	239
11	270	263	241
12	484	344	309
13	315	260	203
14	283	287	212
Mean	286	288	203

<u>Concentration of viable bacteria in the inoculum.</u> Table 4
shows the results of two experiments where explants (variety
9341) were inoculated with different concentrations of
bacteria. For these experiments log phase overnight bacterial
cultures were sedimented and then resuspended by serial
dilution to the appropriate concentration. In each experiment
three separate dilution series of bacteria were prepared from a
common overnight culture. As in the previous experiments 48
explants were prepared in each inoculum, held there for 30
minutes and then cocultured on solid media for three days. The
total number of transformed sectors produced for each
inoculation of 48 explants was recorded. For the first three
inoculation concentrations: 3×10^7, 10^8 and 3×10^8 cells/ml more
transformed sectors were produced by more bacteria. This was
true for both experiments and for every dilution series.

Table 4. Number of transformed sectors produced by inoculation
of soybean hypocotyl explants with different
concentrations of Agrobacterium

Experiment/ Dilution Series	3×10^7	10^8	3×10^8	10^9
1/A	31	96	187	179
1/B	92	176	254	253
1/C	67	156	183	228
2/A	220	349	409	414
2/B	332	448	493	503
2/C	224	386	464	464
Mean	144	269	332	340

At a higher inoculation concentration, 10^9 cells/ml, no
increase in transformation frequency was detected. The mean
number of sectors produced at this concentration did not differ
significantly from that of the concentration below it. The
dose/response profile thus changes at 3×10^8 cell/ml from one
where bacteria are limiting to one where they are saturating.

The absolute number of transformants produced at a given
concentration varies substantially between experiments. Since
a plateau is apparently reached in each experiment the
condition of the Agrobacteria, rather than the numbers of them,
is likely to be the limiting factor. It is also possible that
the different soybean explant preparations varied in their
capacity for transformation. A third possibility is that the
real length of the inoculations varied between experiments.
Though this was unlikely, experiments were conducted to define
an inoculation time period sufficient to saturate a given
concentration. A period of thirty minutes was determined to
produce maximal numbers of events with additional time having
no effect (data not shown).

Sequential inoculation. In an attempt to force the
Agrobacteria to infect the soybean cells, an experiment was
conducted wherein sets of 48 soybean hypocotyl explants

(variety 9341) were inoculated once or twice for 30 minutes each with bacteria at 5x10^8 viable cells/ml. All explants were cocultured for three days. The results are shown in Table 5. The average number of transformed sectors was significantly
5   higher (315) for the 2X treatment than it was for the 1X treatment (286). It appears that additional transformation events may be produced by sequential inoculation. It is believed that true saturation of the pool of transformable soybean cells is not accomplished by a single inoculation and
10 numbers of viable bacteria remain limiting even when inoculations are made at very high concentrations. However, the great majority of associations which lead to transformation occur in the first thirty minute inoculation.

15 Table 5. Effect of sequential inoculation on Agrobacterium mediated transformation of soybean hypocotyl.

	1X	2X
20		275	281
	325	386
	271	251
25		274	341
Mean	286	315

30 Susceptible and resistant soybean genotypes. The results presented in Table 1 showed that all soybean varieties tested can be transformed by Agrobacterium using the method of this invention. To determine the relative susceptibility of soybean varieties to Agrobacterium-mediated transformation matched pair
35 analyses of putative resistant varieties, Corsoy 79 and Century were conducted with variety Peking as the sensitive partner. Inoculations were conducted in petri dishes with stainless steel mesh dividers (0.5 mm pore) so that explants of the different varieties could be prepared in a common inoculum. Twenty-four
40 explants of the 'resistant' varieties were prepared in each of twelve separate petri dishes containing an equal number of 'sensitive' explants. The results are given in Table 6. Variety Peking was transformed at approximately two times the frequency

of variety Century and approximately three times the frequency of
variety Corsoy 79.   While variety dependent susceptibility is
confirmed by these experiments the degree, 2-3 fold, is small
enough as to have no serious implications regarding the use of

5 Agrobacterium in transformation of diverse soybean germplasm.

Table 6. Comparison of susceptibility of selected soybean
genotypes to Agrobacterium-mediated transformation.

		Peking	Century	Peking	Corsoy 79
10					
	1	101	46	76	48
	2	49	49	81	58
	3	105	42	99	34
15	4	29	4	143	41
	5	43	1	131	25
	6	27	7	85	51
	7	13	22	144	17
	8	34	13	84	20
20	9	31	9	192	31
	10	39	16	124	38
	11	19	13	0	0
	12	3	1	3	1
25	Mean	41	19	97	30

Agrobacterium strain.   Two alternate, disarmed Agrobacterium
strains were compared with the octopine strain, LBA4404 for their
30 ability to mediate transformation of soybean hypocotyl cells;
they were the L,L-succinamopine strain EHA 101 and the nopaline
strain C58-pz707.   Table 7 shows the results of an experiment in
which increasing concentrations of each of these strains are used
to inoculate Williams 82 hypocotyl explants.   Forty-eight
35 explants were inoculated with each concentration of each strain.
LBA4404 was included as a control at $3x10^8$ viable cells/ml.   Both
of the strains produced transformed sectors.   In both cases the
transformation frequency was dependent upon the concentration of
bacteria in the inocula, with more bacteria producing more
40 transformation events.   At the levels tested neither gave a
substantial change in transformation as compared with LBA4404.

Table 7. Comparison of transformation of soybean hypocotyl by
selected strains of Agrobacterium tumefaciens.

	Viable cells/ml				
Strain	10^7	3×10^7	10^8	$3 \times 10_8$	10_9
LBA4404	–	–	–	258	–
EHA101	16	38	51	155	274
C58–pZ707	11	13	118	262	–

The following examples illustrate various applications in
accordance with the invention, but are in no way intended to
limit the scope thereof.

Example 1
Soybean (Glycine max) seed, Pioneer variety 9341, was
surface sterilized by exposure to chlorine gas evolved in a
glass bell jar. Gas was produced by adding 3.5 ml hydrochloric
acid (34-37% w/w) to 100 ml sodium hypochlorite (5.25% w/w).
Exposure was for 16-20 hours in a container approximately one
cubic foot in volume.   Surface sterilized seed was stored in
petri dishes at room temperature. Seed was germinated by
plating on 1/10 strength agar solidified medium according to
Gamborg [B5 basal medium with minimal organics, Sigma Chemical
Co., cat. no. G5893, 0.32 gm/L; sucrose, 0.2% w/v and 2-[N-
morpholino]ethanesulfonic acid (MES), 3.0 mM] without plant
growth regulators and culturing at 28°C with a 16 hour day
length and cool white fluorescent illumination of approximately
20 μEm2S1. After three or four days, seed could be prepared
for cocultivation. The seed coat was removed and the elongating
radicle was removed 3-4 mm below the cotyledons. Ten prepared
seed were held in each of several petri dishes.

Example 2
Overnight cultures of Agrobacterium tumefaciens strain LBA
4404 harboring the binary plasmid p12GUSBN17 (DP1816) or p12-4X
(DP1813), grown to log phase in Minimal A medium containing

- 15 -

tetracycline, 1.0 μg/ml, were pooled and an optical density measurement at 550 nm was taken. Sufficient volume of the culture was placed in 15 ml conical centrifuge tubes such that upon sedimentation between 1.0 and 2.0 x 1010 cells were
5 collected in each tube, where O.D.550 1.0= 1.4 x 109 cells/ml. Sedimentation was by centrifugation at 6000 g for 10 minutes. After centrifugation the supernatant was decanted and the tubes were held at room temperature until inoculum was needed but not longer than one hour.
10

Example 3

      Inoculations were conducted in batches such that each plate of seed was treated with a newly resuspended pellet of Agrobacterium. One at a time the pellets were resuspended in 20
15 ml inoculation medium. Inoculation medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v. 6-benzylaminopurine (BAP), 44 μM; indolebutyric acid (IBA), 0.5 μM; acetosyringone (AS), 100 μM and was buffered to pH 5.5 with MES, 10 mM. Resuspension was by vortexing. The inoculum was then poured
20 into a petri dish containing prepared seed and the cotyledonary nodes were macerated with a surgical blade. This was accomplished by dividing seed in half by longitudinal section through the shoot apex preserving the two whole cotyledons. The two halves of the shoot apex were then broken off their
25 respective cotyledons by prying them away with a surgical blade. The cotyledonary node was then macerated with the surgical blade by repeated scoring along the axis of symmetry. Care was taken not to cut entirely through the explant to the abaxial side. Twenty explants were prepared in roughly five
30 minutes and then incubated for 30 minutes at room temperature without agitation. Additional plates were prepared during this time. After 30 minutes the explants were transferred to plates of the same medium solidified with Gelrite (Merck & Co., Inc.), 0.2% w/v. explants were embedded with the adaxial side up and
35 level with the surface of the medium and cultured at 22°C for three days under cool white fluorescent light, approximately 20 μEm^2S^1.

Example 4

After three days the explants were moved to liquid counterselection medium. Counterselection medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v; BAP, 5.0 μM ;
5 IBA, 0.5 μM; vancomycin, 200 μg/ml; cefotaxime, 500 μg/ml and was buffered to ph 5.7 with MES, 3 mM. Ten explants were washed in each petri dish with constant, slow gyratory agitation at room temperature for four days. Counterselection medium was replaced four times.

10

Example 5

The explants were then picked to agarose solidified selection medium. Selection medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0%, w/v; BAP, 5.0 μM; IBA, 0.5
15 μM; kanamycin sulfate, 50 μg/ml; vancomycin, 100 μg/ml; cefotaxime, 30 μg/ml; timentin, 30 μg/ml and was buffered to pH 5.7 with MES, 3.0 mM. Selection medium was solidified with SeaKem agarose, 0.3% w/v. The explants were embedded in the medium, adaxial side down and cultured at 28°C with a 16 hour
20 day length and cool white fluorescent illumination of 60-80 μEm^{2}S^1.

Example 6

After two weeks explants were again washed with liquid
25 medium on the gyrotory shaker. This time the wash was conducted overnight in counterselection medium containing kanamycin sulfate, 50 μg/ml. The following day explants were picked to agarose solidified selection medium. Again they were embedded in the medium, adaxial side down, Culture was as
30 before for another two week period.

Example 7

After one month on selective media transformed tissue became visible as green sectors of regenerating tissue against
35 a background of bleached, less healthy tissue. Explants without green sectors were discarded, explants with green sectors were transferred to elongation medium. Elongation medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0%

w/v; IBA, 3.3 μM; gibberellic acid, 1.7 μM; vancomycin, 100 μg/ml; cefotaxine, 30 μg/ml; and timentin, 30 μg/ml, buffered to pH 5.7 with MES, 3.0 mM. Elongation medium was solidified with gelrite, 0.2% w/v. They were embedded adaxial side up and
5   cultured as before. Culture was continued on this medium with transfers to fresh plates every two weeks. When shoots became 0.5 cm in length they were excised at the base and placed in rooting medium in 13x100 mm test tubes. Rooting medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 15 gm/L;
10 nicotinic acid, 20 μM; pyroglutamic acid (PGA), 900 mg/L and IBA, 10 μM. It was buffered to pH 5.7 with MES, 3.0 mM and solidified with Gelrite, 0.2% w/v. After ten days the shoots were transferred to the same medium without IBA or PGA. Shoots were rooted and held in these tubes under the same
15 environmental conditions as before.

Example 8

      When a root system was well established the plantlet was transferred to sterile soil mix in plant cons (ICN Biomedicals,
20 Inc., cat. no. 26-720 & 1-02). Temperature, photoperiod and light intensity remained the same as before, Under these conditions the regenerants became vigorous, mostly normal (though small) plants. When their root systems again became well established a corner of the plant con was cut off and the
25 plants were gradually hardened off in an environmental chamber or greenhouse. Finally they were potted in soil mix and grown to maturity, bearing seed, in a greenhouse.

- 18 -

CLAIMS

We claim:

5   1. A genotype-independent method for producing a transgenic
soybean plant which comprises:

        a. cocultivating an explant derived from a hypocotyl or
cultured cotyledonary nodes of a germinated soybean seed with
10 Agrobacterium species containing a chimeric gene in the
presence of a signal compound selected from the group
consisting of acetosyringone, α-hydroxyacetosyringone,
acetovanillone, syringaldehyde, syringic acid, and sinapinic
acid and mixtures thereof;

15
        b. maintaining a temperature for cocultivation of from 18
to 28°C; and

        c. inducing virulence of the Agrobacterium by decreasing
20 the pH of the plant culture media below pH 6.0.

    2. A method according to Claim 1 wherein the concentration of
Agrobacterium cells is from 10^8 to 3×10^9 ml.

25 3. A method according to Claim 1 wherein the cocultivation is
carried out by sequential inoculation of Agrobacterium.

    4. A method according to Claim 1 wherein the chimeric gene is
a seed storage protein gene.

30
    5. A method according to Claim 1 wherein the chimeric gene is
the 2S storage protein from Bertholletia excelsa.

    6. A method of regenerating soybean plants from cotyledonary
35 nodes which comprises:

        a. dividing the node;

    b.   culturing the divided node on a nutrient medium
until callus tissue develops which contains shoots; and

    c.   removing the shoots from said callus and rooting the
5 shoots on a nutrient medium containing hormone and pyroglutamic
acid to form a plantlet.

7.   A method according to Claim 6 wherein the rooting medium is
Gamborg medium.
10

8.   A method according to Claim 6 wherein said plantlet is
transferred to a medium comprising soil and allowed to grow
thereon until a whole plant is generated therefrom.